Kidney International, Vol. 57 (2000), pp. 1895–1904

Exposure of endothelial cells to recombinant human erythropoietin induces nitric oxide synthase activity

Debendranath Banerjee, Marilis Rodriguez, Mihir Nag, and John W. Adamson

The Lindsley F. Kimball Research Institute of The New York Blood Center, New York, New York, USA

Exposure of endothelial cells to recombinant human erythropoietin induces nitric oxide synthase activity.

Background. Anemic patients with chronic renal failure receiving recombinant human erythropoietin (rHuEPO) therapy frequently develop hypertension through an unknown mechanism. We hypothesize that EPO receptors (EPORs) on endothelial cells (ECs) in various sites of vasculature may mediate the activities of nitric oxide synthase (NOS) and/or the release of endothelin-1 (ET-1), contributing to blood pressure changes. We tested this hypothesis using primary cultures of ECs obtained from human coronary artery (HCAEC), pulmonary artery (HPAEC), dermis (HDEC), and umbilical vein (HUVEC).

Methods. EPORs were measured by ^{125}I-EPO binding. The effect of EPO on EPOR, ET-1, and NOS mRNA levels was assessed by quantitative reverse transcription-polymerase chain reaction. Cellular NOS activity and ET-1 release into the medium was measured by the NOS*detect* assay and by radioimmunoassay kits.

Results. Short-term (4 h) treatment with EPO (4 U/mL) did not change the number or affinity of EPOR per cell. Neither were there any changes in the amount of EPOR, ET-1, and NOS transcripts (cDNA/μg of mRNA) nor in ET-1 release and NOS activity. In HUVEC only, 24-hour exposure to EPO caused a threefold increase in NOS transcript. In other cells, EPO treatment for six days increased NOS activity by twofold to fourfold.

Conclusions. We show that upon extended exposure, EPO induces NOS activity but does not affect ET-1 release. These findings indicate that the hypertensive effect of EPO is not likely to be caused by a direct effect on ECs.

Erythropoietin (EPO) is a glycoprotein hormone that is the primary regulator of erythropoiesis [1]. EPO is produced by the kidney in adults and by the liver in fetal life [1, 2]. EPO functions through its interaction with a single chain cell surface receptor of the cytokine receptor superfamily [3, 4]. The EPO receptor (EPOR) on erythroid progenitor cells is the primary target for EPO binding [5, 6].

In hematopoietic cells, EPOR mRNA is expressed at moderate levels [7]. EPOR or EPOR mRNA are also expressed on nonhematopoietic cells, including human umbilical vein endothelial cell (HUVEC) [8, 9], rat brain capillary endothelial cells (ECs) [10], murine hippocampal and cerobrocortical areas [11], and primary cultured hippocampal and cortical neurons [12, 13]. The EPORs are functional in HUVEC and mouse brain.

The angiogenic effect of EPO has been studied in ovariectomized mice in which an injection of EPO into the uterine cavity promoted blood vessel formation in the endometrium [14]. EPO stimulates proliferation and migration of human and bovine EC and also angiogenesis of the rat thoracic aorta [8, 15].

Many anemic patients with chronic renal failure receive treatment with recombinant human erythropoietin (rHuEPO) with beneficial results. The major side-effect of this treatment is the development of clinically significant hypertension [16–18]. Several factors have been implicated in rHuEPO-associated hypertension: an increase in hematocrit and red blood cell mass resulting in increased blood viscosity [19], a loss of hypoxic vasodilation [20], a direct vasoconstrictor effect [21], and an increase in calcium uptake by vascular smooth muscle cells [22, 23]. Nevertheless, the principal cause of EPO-induced hypertension is unknown. The angiogenic nature of EPO and the occurrence of EPOR on ECs suggest that EPO may directly stimulate ECs.

In humans, clinical events such as hypertension and thrombosis are often localized in specific vessels. This pattern, in part, may be due to the heterogeneity of EC themselves [reviewed in 24, 25]. Since the discovery of EC-dependent vasodilation by Furchgott and Zawadzki, ECs have been recognized as an important functional unit. Upon induction by vasoactive agents, such as acetylcholine and bradykinin, ECs secrete short-lived relaxing factor(s) causing relaxation of underlying smooth muscle cells [reviewed in 26, 27]. One endothelium-derived relaxing factor is nitric oxide (NO) [28]. EC-dependent vaso-

Key words: erythropoietin, receptor, endothelin, nitric oxide, anemia, chronic renal failure.

Received for publication August 5, 1999
and in revised form October 20, 1999
Accepted for publication December 2, 1999

constriction has also been observed in response to various physiological stimuli such as thrombin [29], hypoxia [30], and mechanical stretch [31]. Endothelin-1 (ET-1) is the most potent vasoconstrictor peptide produced by ECs [32, 33]. Vascular smooth muscle cells and glomerular mesangial cells respond to ET-1 by cellular contraction and proliferation [34, 35]. To date, very little information is available on the effect of EPO on endothelium from different vascular sites.

In this study, we obtained primary cultures of EC prepared from various sites of human vasculature and examined the levels of EPO binding to the cells and the effect of EPO on EPOR, ET-1, and NO synthase (NOS) mRNA expression by quantitative reverse transcription-polymerase chain reaction (RT-PCR). We also measured the effect of EPO on ET-1 release into the culture medium and endothelial NOS enzyme activities. We found that EPORs are present on all ECs tested and that EPOR, ET-1, and NOS mRNA levels are unaffected by treatment with EPO. However, the extended exposure of vascular ECs to EPO at super-physiological concentrations induced NOS activity, which may contribute to the regulation of blood pressure.

METHODS

Cells and cell culture

Primary cultures of human ECs, prepared from umbilical vein (HUVEC), coronary artery (HCAEC), pulmonary artery (HPAEC), and dermis (HDEC) were purchased from Clonetics (San Diego, CA, USA). Cells were cultured at 37°C in modified MCDB 131 medium (Clonetics) supplemented with 5% fetal bovine serum (FBS; Hyclone Laboratories, Logan, UT, USA) in an air-5% CO_2 atmosphere at constant humidity. Cells were maintained in continuous culture and used within three to five passages.

^{125}I-EPO binding reactions

For binding studies (3-[^{125}I]iodotyrosyl)rHuEPO (Amersham Corp., Arlington Heights, IL, USA) was used. The experimental procedures for the binding of ^{125}I-EPO to ECs have been reported elsewhere [8]. Briefly, confluent monolayers of ECs (1×10^6 cells/well) were washed with binding medium (0.05 mol/L phosphate buffer, pH 7.4, containing 150 mmol/L NaCl, 68 mmol/L $CaCl_2$, 50 mmol/L $MgCl_2$, and 1 mg/mL human serum albumin) and incubated at 22°C with 1.0 mL binding buffer containing 60,000 counts per min (cpm) of ^{125}I-EPO (Sp. Act. 300 to 900 Ci/mmol). At the end of specified time of incubation, wells were gently washed three times with prewarmed phosphate-buffered saline (PBS) containing 1% FBS, and the cells were then solubilized in lysis buffer [20 mmol/L HEPES, pH 7.4, 1% Triton X-100, 10% (vol/vol) glycerol, and 0.1 mg/mL of bovine serum albumin]. The radioactivity of the lysates was measured in a gamma counter. Nonspecific binding was assessed in the presence of a 100-fold molar excess of unlabeled EPO added at the start of incubation and subtracted from the total to calculate net specific binding.

Preparation of RNA

Approximately 1 to 5×10^7 cells, either treated with rHuEPO (4 U/mL) for four hours or untreated, were washed twice with PBS, and RNA was extracted with TRIzol reagent as recommended by the supplier (Life Technologies, Grand Island, NY, USA). mRNA was adsorbed onto oligo(dT)-cellulose columns (Qiagen, Valencia, CA, USA). The total amount and concentration of mRNA were determined spectrophotometrically.

Reverse transcription of RNA and synthesis of cDNA

An aliquot of mRNA (0.5 to 1 μg) was incubated at 42°C for one hour in 20 μL of 10 mmol/L Tris-HCl, pH 8.8, containing 50 mmol/L KCl, 0.1% Triton X-100, 5 mmol/L $MgCl_2$, 1 mmol/L each dNTPs, 20 units of RNAsin, and 0.5 μg oligo dT_{15} primer. The cDNA was synthesized by primer extension using 15 units of avian myeloblastosis virus (AMV) reverse transcriptase per μg of RNA (Promega, Madison, WI, USA).

Preparation of internal standard DNA and amplification of cDNA

An internal standard was constructed by PCR amplification of genomic DNA using the following primer sets: (*1*) 5′-GCACCGAGTGTGTGCTGAGC-3′ and 5′-GGTCAGCAGCACCAGGATGA-3′ for hEPOR [36], (*2*) 5′-CCGTATGGACTTGGAAGCCC-3′ and 5′-CTGGTTTGTCTTAGGTGTTCC-3′ for ET-1 [37], and (*3*) 5′-CCTCCCCCCGGCTGGGTGCG-3′ and 5′-GCACCTCCAGAAGCGTGGG-3′ for NOS [38]. The same primer sets were used for cDNA amplification.

Quantitative polymerase chain reaction

To quantitate gene-specific mRNA, multiple reaction mixtures were prepared with known amounts of cDNA. Serial dilutions of internal standard cDNA and [α-^{32}P]CTPwere added to each tube and coamplified. The products were analyzed by gel electrophoresis. The bands representing gene-specific cDNA and the standard DNA fragment were recovered and counted, and the results were plotted as cpm versus the concentration of external standard.

Endothelin-1 assay

The medium from cultured ECs, either exposed for four hours to rHuEPO (4 U/mL) or untreated, was harvested and centrifuged at $500 \times g$ at 4°C for 10 minutes. The recovered supernatants were then stored at −70°C. ET-1 determinations were performed using an ET-1 radioimmunoassay kit (Peninsula Lab., Belmont, CA, USA).

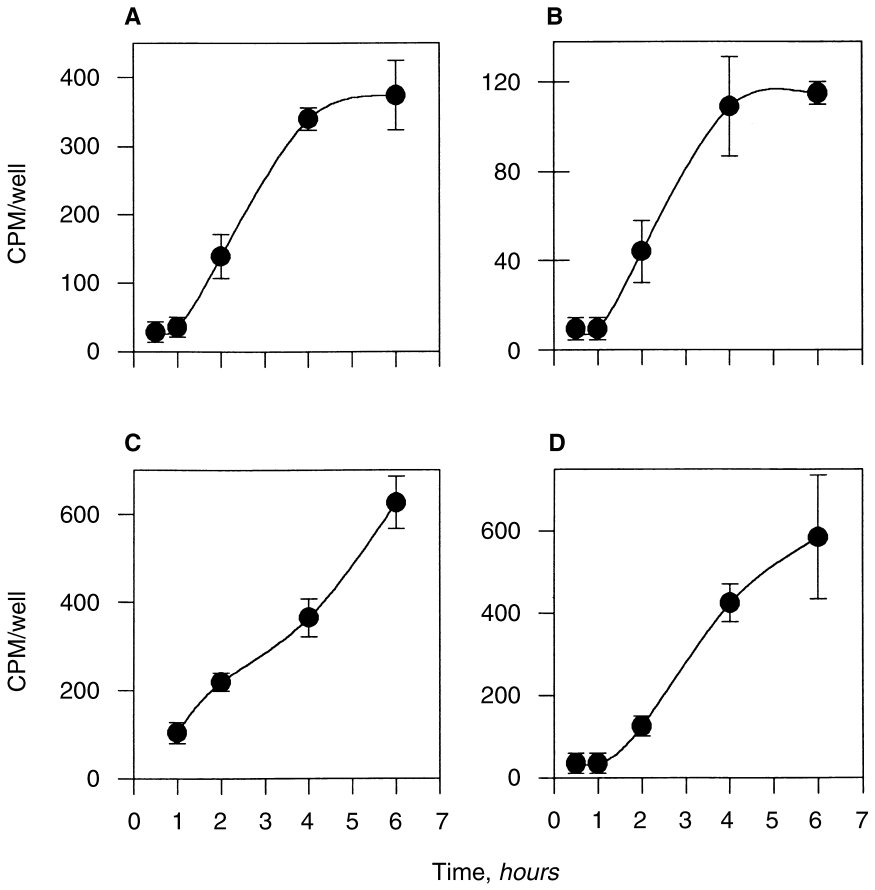

Fig. 1. Kinetics of ^{125}I-recombinant human erythropoietin (rHuEPO) binding to endothelial cells. Monolayers of human endotheial cells prepared from (*A*) umbilical vein (HUVEC), (*B*) coronary artery (HCAEC), (*C*) dermis (HDEC), and (*D*) pulmonary artery (HPAEC) were incubated with 60,000 cpm of ^{125}I-EPO (Sp. act. 166 Ci/mmol) in 1.0 mL phosphate buffered saline (PBS) containing $CaCl_2$ and $MgCl_2$ for increasing time intervals at 22°C. At the end of each time point, cells were washed, and the specific radioactivity associated with each well was determined as described in the **Methods** section. The results from three independent experiments are shown. Error bars represent standard errors ($N = 3$).

Nitric oxide synthase assay

Confluent monolayers of ECs, either treated with rHuEPO (4 U/mL) daily for six days or untreated, were washed twice with PBS, harvested, and homogenized. The homogenate was centrifuged, and the supernatant protein concentration was adjusted to 1 mg/mL. The NOS activity was measured using a NOS*detect* assay kit according to the supplier's specification (Stratagene, La Jolla, CA, USA).

RESULTS

^{125}I-EPO binding study

To compare binding of ^{125}I-EPO to EC from various vascular sites, cells were exposed to ^{125}I-EPO with or without a 100-fold excess of unlabeled EPO for increasing time intervals. The time dependence of binding of ^{125}I-rHuEPO to ECs from various sources is shown in Figure 1. All ECs readily bound ^{125}I-EPO, and the specific binding increased with time, reaching a plateau in about four hours for HUVEC and HCAEC. The plateauing time was slightly longer (approximately 6 h) for HDEC and HPAEC. The fluctuation of increase binding in various ECs may correspond to the differences in the internalization and degradation of EPO as the binding assays were performed at 22°C. The concentration dependence of EPO binding was measured over the range from 1 to 18 nmol/L (Fig. 2). Specific binding was found to be saturable. The mean number of EPOR per cell was calculated by Scatchard plot analysis (Fig. 3). The calculated number of binding sites was 44,668 for HUVEC, 49,845 for HCAEC, 56,106 for HDEC, and 58,093 for HPAEC, while the dissociation constants (K_d) were 5.6×10^{-9} mol/L for HUVEC, 7.7×10^{-9} mol/L for HCAEC, 1.10×10^{-8} mol/L for HDEC, and 1.50×10^{-8} mol/L for HPAEC. Exposure of cells for two days to EPO resulted in no significant change in the number of receptor sites (data not shown).

Quantitation of mRNA expression

To confirm that EPO binding correlated with EPOR gene expression, ECs were cultured with or without EPO for four hours, and EPOR mRNA was measured by quantitative RT-PCR. The results are shown in Figure 4. The expected 197 bp cDNA fragment and 285 bp internal standard DNA were amplified from ECs from all sources. Further cloning and sequencing confirmed that the 197 bp fragment represented hEPOR mRNA.

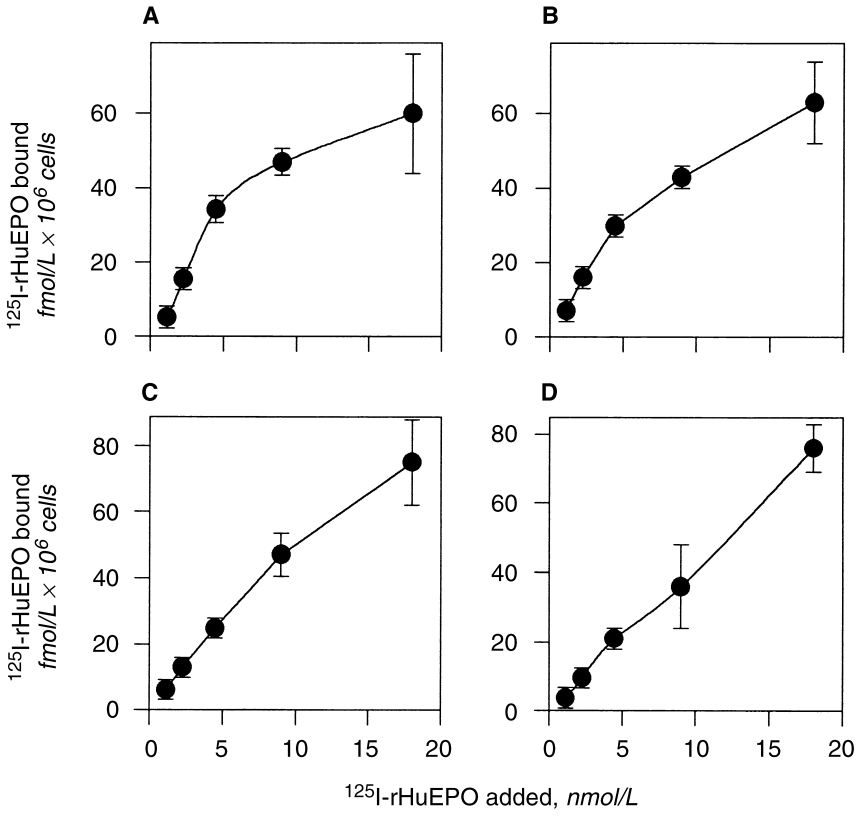

Fig. 2. Dose response of ^{125}I-EPO binding to endothelial cells. Confluent monolayers of (*A*) HUVEC, (*B*) HCAEC, (*C*) HDEC, and (*D*) HPAEC were washed and incubated with increasing amounts of ^{125}I-EPO (1 to 18 pmol) for four hours at 22°C. Monolayers were washed, and the specific radioactivity associated with each well was determined as described in the **Methods** section. The results from three independent experiments are shown. Error bars represent standard errors ($N = 3$).

The amount of hEPOR-specific cDNA/μg of mRNA determined in duplicate by quantitative PCR was 5.1×10^{-4} ng for HUVEC, 11.0×10^{-4} ng for HPAEC, 4.0×10^{-4} ng for HCAEC, and 64.0×10^{-4} for HDEC. With the exception of HCAEC, treatment of these cells with EPO for up to four hours had little or no effect on EPOR mRNA. In contrast, a twofold increase of EPOR mRNA was measured in EPO-treated HCAEC culture (Table 1). Treatment of HUVEC cells with EPO for 1, 2, 4, 6, and 24 hours had little or no effect on EPOR mRNA (data not shown).

Effects of rHuEPO on ET-1 gene expression and ET-1 secretion

A direct link between ET-1 and EPO has been established from the findings that HUVECs have EPOR [8, 9] and that rHuEPO increases ET-1 release by bovine pulmonary artery ECs [39]. We therefore examined the effect of EPO on ET-1 expression by cultured HUVEC, HCAEC, HPAEC, and HDEC. As shown in Figure 5, a 290 bp ET-1–specific partial cDNA fragment and a 450 bp internal standard DNA were amplified by ET-1 primer sets and RT-PCR. The 290 bp fragment was cloned, and its identity confirmed by sequencing. The quantitative values are shown in Table 2. The amount of ET-1–specific cDNA/μg of mRNA in duplicate quantitative PCR was 2.8×10^{-2} ng for HUVEC, 3.8×10^{-2} ng for HPAEC, 2.6×10^{-2} ng for HCAEC, and 0.34×10^{-2} ng for HDEC. These values were unchanged in HCAEC, HPAEC, and HDEC cultures exposed to rHuEPO. However, the level of ET-1 transcript rose twofold in EPO-treated HUVEC cultures. Treatment of HUVEC cells with EPO for 1, 2, 4, 6, and 24 hours had no significant effect on ET-1 mRNA (data not shown).

Table 3 shows ET-1 release by cultured HUVEC, HCAEC, HPAEC, and HDEC during a four-hour treatment with 4 U/mL rHuEPO. All cells released ET-1 into the media. About 35 to 48 pg of ET-1 were released by 2×10^5 cells. These values also did not change with EPO treatment, consistent with the results shown in Table 2.

Effect of rHuEPO on NOS activity

Nitric oxide is thought to play a central role in vascular homeostsis. NO, a heterodiatomic free radical product, is generated through the oxidation of L-arginine to L-citrulline by NOS. NO has been demonstrated to inhibit thrombosis, cytokine-induced vascular cell adhesion molecule-1 (VCAM) expression, leukocyte adhesion to endothelium, and smooth muscle proliferation and migration [40–42]. To investigate the effect of EPO on NOS activity, we measured NOS mRNA levels by RT-PCR. The results are shown in Figure 6. Partial cDNA fragments (approximately 230 bp) and standard DNA (approximately 500 bp) were isolated, cloned, and char-

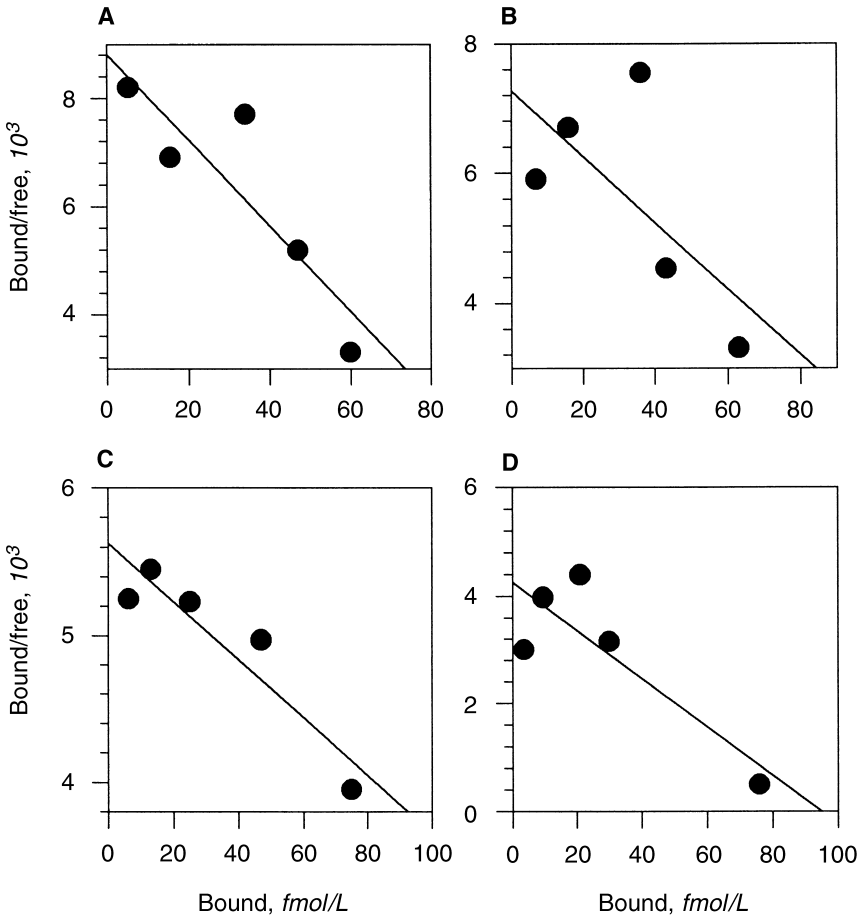

Fig. 3. Scatchard analysis of ^{125}I-EPO binding to endothelial cells. Plots represent Scatchard analyses of binding data shown in Figure 2. The data suggest the presence of a single class receptor with different affinities in different cell types. For (*A*) HUVEC, $K_d = 5.6 \times 10^{-9}$ mol/L and 44,668 receptor sites per cell; (*B*) HCAEC, $K_d = 7.7 \times 10^{-9}$ mol/L and 49,845 receptor sites per cell; (*C*) HDEC, $K_d = 1.10 \times 10^{-8}$ mol/L and 56,106 receptor sites per cell; (*D*) HPAEC, $K_d = 1.5 \times 10^{-8}$ mol/L and 58,093 receptor sites per cell.

acterized by sequencing. The quantitative values are shown in Table 4. The NOS-specific mRNA in ECs varied from 1.0×10^{-3} to 1.8×10^{-3} ng/μg of mRNA. Exposure of the cells to rHuEPO had little effect. However, the presence of EPO for 24 hours induced a threefold increase in NOS-specific mRNA (5.4×10^{-3} ng cDNA/μg of mRNA) compared with control cultures of HUVEC (1.8×10^{-3} ng cDNA/μg of mRNA). The increase values were maintained for up to six days upon daily removal and replenishment with fresh EPO-supplemented medium. Short-term exposure for one, two, four, and six hours to EPO, HUVEC exhibited no effect on NOS-specific mRNA (data not shown).

To confirm that the increased NOS-specific mRNA resulted in increased NOS activity, we determined the effect of EPO on NOS activity. As shown in Table 5, long-term EPO treatment resulted in a twofold to fourfold increase in NOS activity in all ECs.

DISCUSSION

The present study demonstrates that ECs prepared from various regions of the vascular tree bind EPO in a dose-dependent manner. The scattering of data points at a low ligand concentration in Scatchard analysis indicates the possibility of more than one binding site. Our finding of a large number of relatively low-affinity binding sites on all EC types tested (Fig. 3) is consistent with the findings of Anagnostou et al [8]. This is in contrast with earlier studies that measured the binding of EPO to its receptors and showed that erythroid progenitor cells have only a small number of cell surface receptors [43–45]. The low number of receptors per cell for growth factors is common in hematopoietic cells [46].

The ligand affinity of EPOR expressed on EC is much lower than that on erythroid cells; the K_d for EPO binding to EPOR on EC varies from approximately 5 to 15 nmol/L [8, 10], while the K_d for the high-affinity receptor on erythroid cells is approximately 50 pmol/L [47]. The reason for the expression of only the low-affinity receptor on ECs is unclear. Expression of only the low-affinity receptor might be crucial to prevent unregulated angiogenesis by endogenous EPO. The presence of EPOR on ECs may originate from the close relationship between vasculogenesis and hematopoiesis in early development [48] and between angiogenesis and hematopoiesis later in development [49].

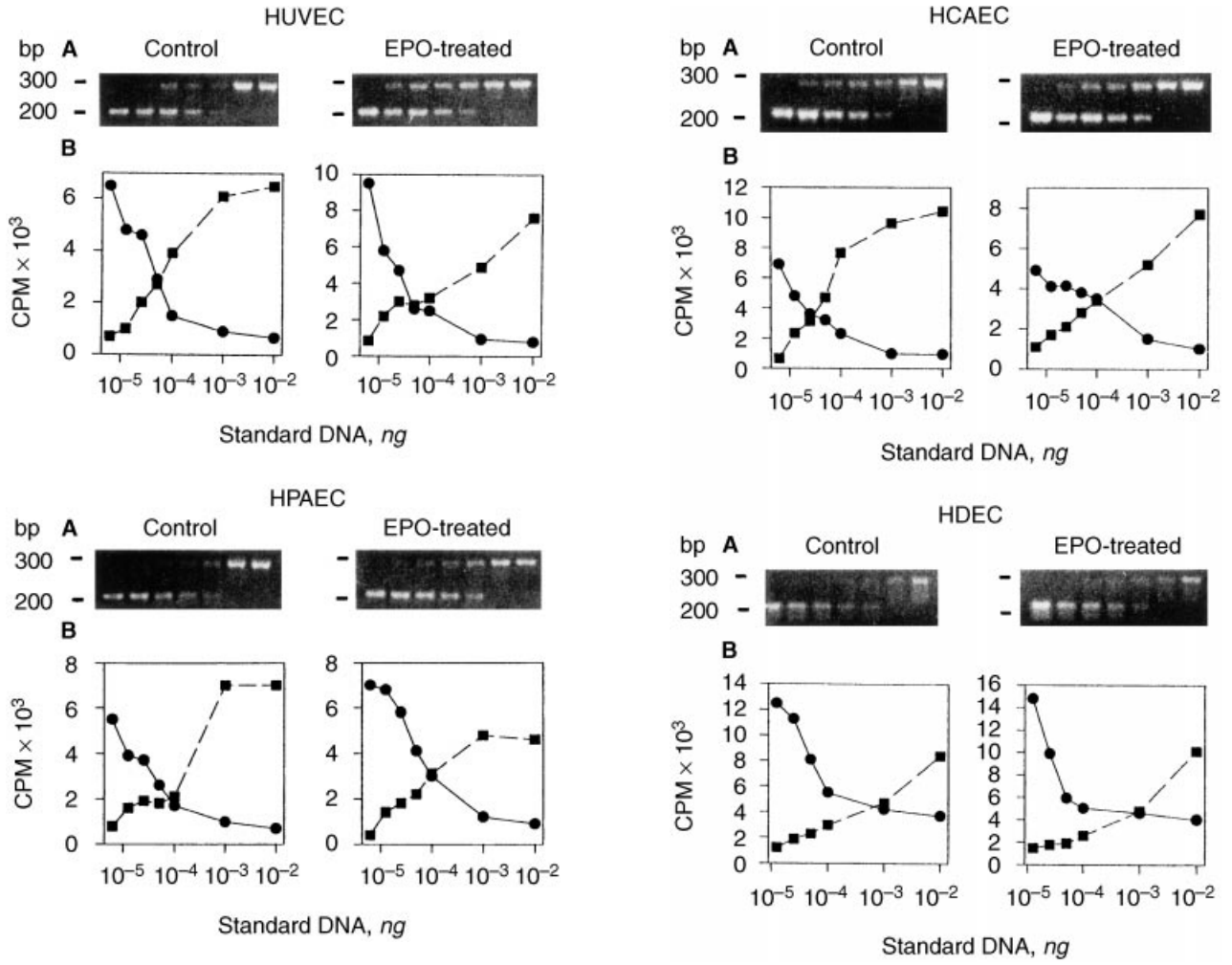

Fig. 4. Polymerase chain reaction (PCR) analysis of human erythropoietin receptor (hEPOR) transcripts in control and EPO-treated HUVEC, HPAEC, HDEC, and HCAEC. Monolayers of HUVEC, HCAEC, HDEC, and HPAEC were incubated with EPO (4 U/mL) for four hours or were untreated, and mRNA was prepared as described in the **Methods** section. cDNA was reverse transcribed from mRNA isolated from respective endothelial cells. Multiple reactions for hEPOR-specific PCR amplification of cDNA were carried out with hEPOR primers. Prior to amplification, increasing amounts of standard DNA and [α-^{32}P]dCTP were added to each reaction. (*A*) The amplification products were resolved by agarose gel electrophoresis. Left panel show control gels and right panels show EPO-treated gels (upper band 285 bp standard DNA and lower band 197 bp hEPOR cDNA; bp, size markers). (*B*) For quantitation, the hEPOR and the standard DNA bands were cut, the radioactivity present in each band was determined and plotted against the amount of standard added to each reaction mixture. Symbols are: (●) standard DNA; (■) hEPOR-specific PCR product. The amount of standard corresponding to the point at which the two PCR products are equal is an indication of the amount of hEPOR cDNA present.

Table 1. Quantitation of human erythropoietin receptor (hEPOR)-specific mRNA expression

	Control	EPO-treated
ECs	*ng cDNA/μg of mRNA*	
HUVEC	5.1×10^{-4}	5.5×10^{-4}
HPAEC	11×10^{-4}	9.2×10^{-4}
HCAEC	4.0×10^{-4}	9.0×10^{-4}
HDEC	64.0×10^{-4}	88.0×10^{-4}

Abbreviations are: ECs, endothelial cells; EPO, erythropoietin; HUVEC, human umbilical vein endothelial cells; HPAEC, human pulmonary artery endothelial cells; HCAEC, human coronary artery endothelial cells; HDEC, human dermis endothelial cells. The values of human EPO receptor-specific mRNA were obtained from Figure 4B. Each sample was assayed three times from two separate experiments.

Compared with OCI-M1 or other human erythroid cell lines, HUVECs have many more EPOR and a lower expression of EPOR mRNA [9]. In our study, the mean number of EPOR per cell varied from 44,668 to 58,093, but the quantity of EPOR mRNA was less than the amount reported for OCI-M1 cells. The significance of this discrepancy is not clear. In erythroid cells, less than 5% of the newly synthesized EPORs are found on the cell surface, and the rest are degraded in the endoplasmic reticulum [50, 51].

Quantitative determination of mRNA expression failed to detect any increase in EPOR gene expression in EPO-treated HUVEC, HPAEC, and HDEC cultures. In contrast, a twofold increase of EPOR mRNA was

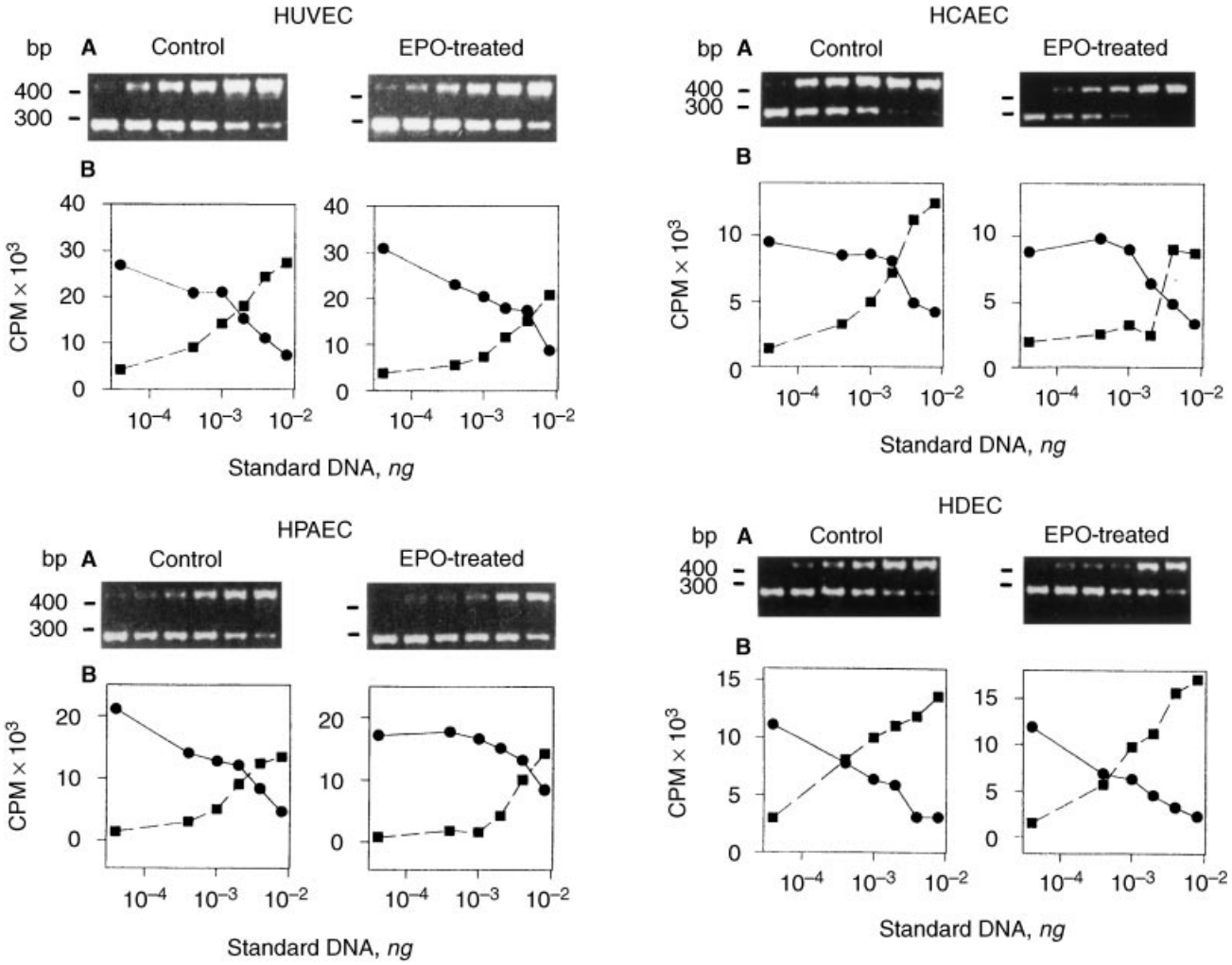

Fig. 5. Polymerase chain reaction (PCR) analysis of endothelin-1 (ET-1) transcripts in untreated and EPO-treated HUVEC, HPAEC, HDEC, and HCAEC. Monolayers of HUVEC, HCAEC, HDEC, and HPAEC cells were either incubated with EPO (4 U/mL) for four hours or were untreated, and mRNA was prepared as described in the **Methods** section. Reverse transcription of total RNA isolated from respective endothelial cells with ET-1–specific primers and multiple reactions for PCR amplification of cDNA were carried out with ET-1 primers and standard DNA as described in Figure 4. (*A*) The amplification products were resolved by agarose gel electrophoresis. Left panels show controls and right panels show EPO treated (upper band 450 bp standard DNA and lower band 290 bp ET-1 cDNA; bp, size markers). (*B*) Quantitation was achieved as described in Figure 4. ET-1 cDNA and the standard DNA bands were cut, the radioactivity present in each band was determined and plotted against the amount of standard added to each reaction mixture. Symbols are: (●) standard DNA; (■) ET-1-specific PCR product to each reaction mixture.

Table 2. Quantitation of endothelin-1 (ET-1)-specific mRNA expression

	Control	EPO-treated
ECs	*ng cDNA/μg of mRNA*	
HUVEC	2.8×10^{-2}	6.2×10^{-2}
HPAEC	3.8×10^{-2}	4.7×10^{-2}
HCAEC	2.6×10^{-2}	2.7×10^{-2}
HDEC	0.34×10^{-2}	0.43×10^{-2}

The values of human ET-1-specific mRNA were derived from Figure 5B. Each sample was assayed three times from two separate experiments. Abbreviations are in Table 1.

Table 3. Effect of EPO on ET-1 production by endothelial cells from various sources

	Control	EPO-treated
ECs	Endothelin-1 *pg/2 × 10⁵ cells*	
HUVEC	35	37
HPAEC	39	36
HCAEC	48	50
HDEC	45	47

HUVEC, HPAEC, HCAEC, and HDEC were incubated in serum-free endothelial cell basal media (EBM) with 4 U/mL rHuEPO for 4 hours. Control cells were exposed to rHuEPO vehicle alone. Results represent the mean of two experiments performed in duplicate. Abbreviations are in Table 1.

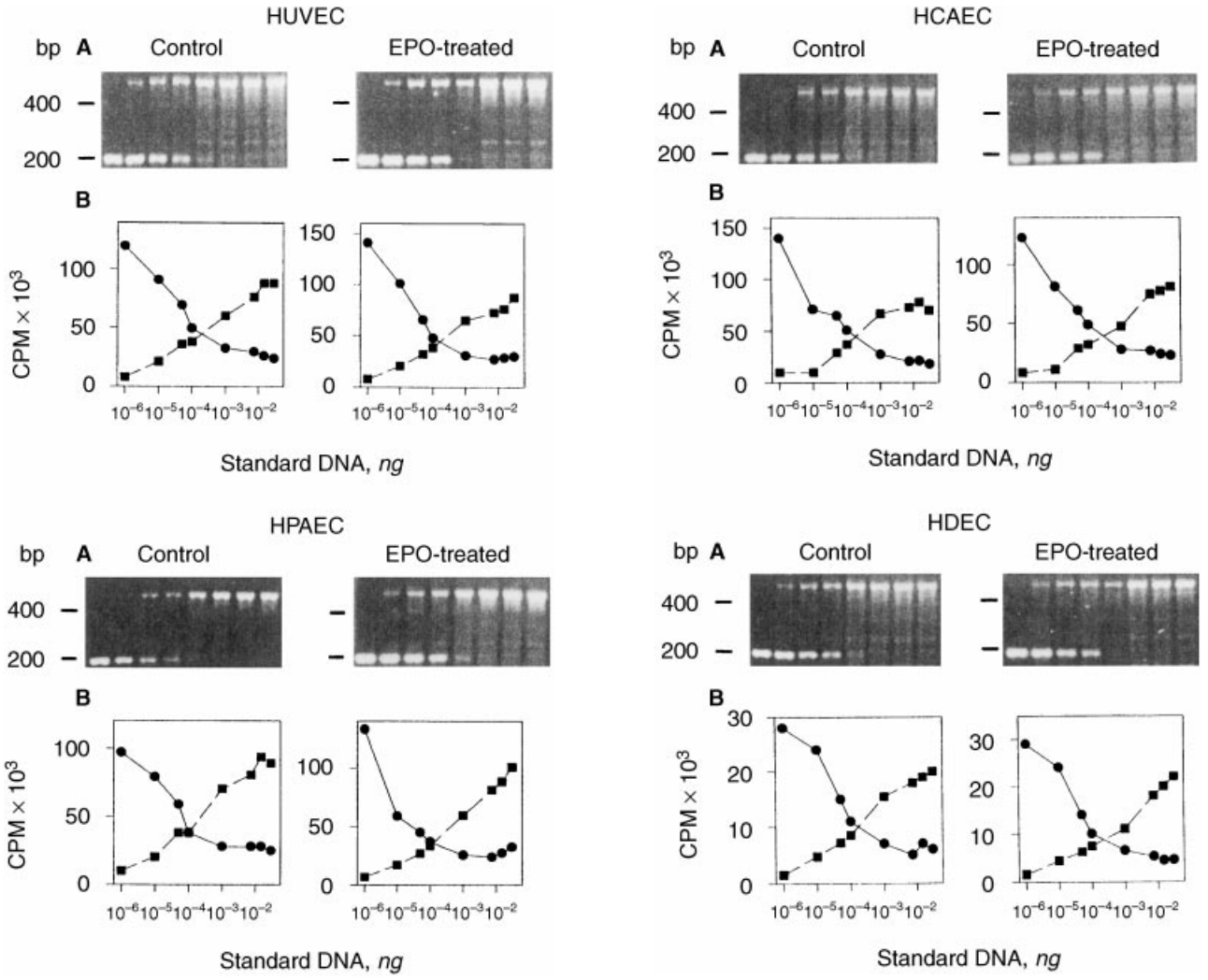

Fig. 6. Polymerase chain reaction (PCR) analysis of nitric oxide synthase (NOS) transcripts in EPO-treated and nontreated HUVEC, HPAEC, HDEC, and HCAEC. Monolayers of HUVEC, HCAEC, HDEC, and HPAEC were either incubated with EPO (4 U/mL) for four hours or were untreated, and mRNA was prepared as described in the **Methods** section. Reverse transcription of total RNA isolated from respective endothelial cells with NOS-specific primers and multiple reactions for PCR amplification of cDNA were carried out with NOS primers and standard DNA, as described in Figure 5. (*A*) The amplification products were analyzed by agarose gel electrophoresis. Left panels show controls and right panels show EPO treated (upper 500 bp band standard DNA and lower 200 bp NOS cDNA band; bp, size markers). (*B*) For quantitation, the NOS and the standard bands were cut, the radioactivity present in each band was determined and plotted against the amount of standard added to each reaction mixture. Symbols are: (●) standard DNA; (■) NOS-specific PCR product.

Table 4. Quantitation of nitric oxide synthase (NOS)-specific mRNA expression

	Control	EPO-treated
ECs	*ng cDNA/μg of mRNA*	
HUVEC	1.8×10^{-3}	1.6×10^{-3}
HPAEC	1.0×10^{-3}	0.8×10^{-3}
HCAEC	1.8×10^{-3}	1.9×10^{-3}
HDEC	1.2×10^{-3}	1.5×10^{-3}

The values of human NOS-specific mRNA were obtained from Figure 6B. Each sample was assayed three times from two separate experiments. Abbreviations are in Table 1.

Table 5. Effect of EPO on NOS activity by endothelial cells from various sources

	Control	EPO-treated	Change(-fold)
ECs	Nitric oxide synthase *fmol ^{3}H-citrulline/min/mg protein*		
HUVEC	30	78	2.6
HPAEC	14	63	4.5
HDEC	30	110	3.6

HUVEC, HPAEC, and HDEC were incubated in complete media (EGM-2 MV) with 4 U/mL rHuEPO. The medium was changed daily for up to 6 days. Control cells were exposed to complete media alone. The cell extracts were prepared and NOS activity was measured as described in the **Methods** section. Results represent the mean of two experiments performed in duplicate. Abbreviations are in Table 1.

found in EPO-treated HCAEC culture (Table 1). Similar determinations of ET-1 transcripts revealed no increase in ET-1 mRNA in EPO-treated HPAEC, HCAEC, and HDEC cultures, whereas a twofold increase of ET-1 mRNA was found in EPO-treated HUVEC (Table 2). The reasons for these differences are not clear. It is possible that the untreated HCAEC and HUVEC cultures contained fewer ECs, and thus, the technique can be limited by its inability to distinguish heterogeneous cell populations.

Normally, ECs contribute to the regulation of blood pressure and blood flow by releasing vasodilators such as NO and prostacyclin (PGI_2), as well as vasoconstrictors including ET-1 and platelet activating factor (PAF) [reviewed in 52]. ECs synthesize ET-1 [53]. ET-1 is not stored in granules but is transcribed after stimulation by hypoxia, shear stress, or ischemia [54]. rHuEPO has been shown to have a direct stimulatory effect on ET-1 release from cultured bovine PAEC [39]. This stimulatory effect was time dependent, peaked at 4 hours, and reached a plateau at 12 hours. In contrast, we saw no effect on ET-1 release in response to EPO in any of the four human EC types in culture. This may be due to differences in culture conditions.

Endothelial cells produce NO, a free radical generated through the oxidation of L-arginine to L-citrulline by NO synthase [55]. One isoform, eNOS, is constitutively active in EC and can be further stimulated by thrombin, bradykinin, or shear stress [56]. Our study demonstrates that short-term treatment of ECs in culture has little or no effect on NOS transcript. However, EC cultures exposed to EPO for several days increased their production of NO. Although we did not measure NO release into the medium and it is impossible to directly extrapolate these results to the in vivo situation, the increased production of NO may contribute to the regulation of blood pressure.

Heidenreich, Rahn, and Zidek [21] and Carlini et al [39] have shown that rHuEPO has a direct vasoconstrictive effect. In addition, in vivo studies of Buemi, Allegra, and Frisina [57] and the results of the present studies (Table 5) are consistent with a vasodilatation effects of EPO. EPO may act by modifying the balance of vasoconstrictive and vasodilatory effects to favor vasoconstriction in the setting of renal failure. Why hypertension is only seen in this patient group remains unresolved.

ACKNOWLEDGMENTS

We wish to thank Mr. Tellervo Huima and Ms. Yelena Oksov for the preparation of the illustrations.

Reprint requests to Debendranath Banerjee, Ph.D., The Lindsley F. Kimball Research Institute of The New York Blood Center, New York, New York 10021, USA.
E-mail: dbanerje@nybc.org

REFERENCES

1. Krantz SB: Erythropoietin. *Blood* 77:419–434, 1991
2. Jelkmann W: Erythropoietin: Structure, control of production, and function. *Physiol Rev* 72:449–489, 1992
3. Bazan JF: Structural design and molecular evolution of a cytokine receptor superfamily. *Proc Natl Acad Sci USA* 87:6934–6938, 1990
4. D'Andrea AD, Lodish HF, Wong GG: Expression cloning of the murine eythropoietin receptor. *Cell* 57:277–285, 1989
5. Wu H, Liu X, Jaenisch R, Lodish HF: Generation of committed erythroid BFU-E and CFU-E progenitors does not require erythropoietin or the erythropoietin receptor. *Cell* 83:59–67, 1995
6. Lin CS, Lim SK, D'Agati V, Costantini F: Differential effects of an erythropoietin receptor gene disruption on primitive and definitive erythropoiesis. *Genes Dev* 10:154–164, 1996
7. Orlic D, Anderson S, Biesecker LG, Sorrentino BP, Bodine DM: Pluripotent hematopoietic stem cells contain high levels of mRNA for c-kit, GATA-2, p45, NF-E2, and c-myb and low levels or no mRNA for c-fms and the receptors for granulocyte colony-stimulating factor and interleukins 5 and 7. *Proc Natl Acad Sci USA* 92:4601–4605, 1995
8. Anagnostou A, Lee ES, Kessimian N, Levinson R, Steiner M: Erythropoietin has a mitogenic and positive chemotactic effect on endothelial cells. *Proc Natl Acad Sci USA* 87:5978–5982, 1990
9. Anagnostou A, Liu Z, Steiner M, Chin K, Lee ES, Kessimian N, Noguchi CT: Erythropoietin receptor mRNA expression in human endothelial cells. *Proc Natl Acad Sci USA* 91:3974–3978, 1994
10. Yamaji R, Okada T, Moriya M, Naito M, Tsuruo T, Miyatake K, Nakano Y: Brain capillary endothelial cells express two forms of erythropoietin receptor mRNA. *Eur J Biochem* 239:494–500, 1996
11. Morishita E, Masuda M, Nagao M, Yasuda Y, Sasaki, R: Erythropoietin receptor is expressed in rat hippocampal and cerebral cortical neurons, and erythropoietin prevents in vitro glutamate-induced neuronal death. *Neuroscience* 76:105–116, 1997
12. Morishita E, Narita H, Nishida M, Kawashima N, Yamagishi K, Masuda S, Nagao M, Hatta H, Sasaki R: Anti-erythropoietin receptor monoclonal antibody: Epitope mapping, quantification of the soluble receptor and detection of the solubilized transmembrane receptor and the receptor expressing cells. *Blood* 88:465–471, 1996
13. Digicaylioglu M, Bichet S, Marti HH, Wenger RH, Rivas LA, Bauer C, Gassmann M: Localization of specific erythropoietin binding sites in defined areas of the mouse brain. *Proc Natl Acad Sci USA* 92:3717–3720, 1995
14. Yasuda Y, Masuda S, Chikuma M, Inoue K, Nagao M, Sasaki R: Estrogen-dependent production of erythropoietin in uterus and its implication in uterine angiogenesis. *J Biol Chem* 273:25381–25387, 1998
15. Carlini RG, Reyes AA, Rothstein M: Recombinant human erythropoietin stimulates angiogenesis in vitro. *Kidney Int* 47:740–745, 1995
16. Bode-Boger SM, Bodger RH, Kuhn M, Radermacher J, Frolich JC: Endothelin release and shift in prostaglandin balance are involved in the modulation of vascular tone by recombinant erythropoietin. *J Cardiovasc Pharmacol* 20:25–28, 1992
17. Caravaca F, Pizarro JL, Arrobas M, Cubero JJ, Garcia MC, Perez-Miranda M: Antiplatelet therapy and development of hypertension induced by recombinant human erythropoietin in uremic patients. *Kidney Int* 45:845–851, 1994
18. Raine A, Roger S: Effects of erythropoietin on blood pressure. *Am J Kidney Dis* 18:76–83, 1991
19. Steffen HM, Brunner R, Muller R, Degenhardt S, Pollok M, Lang R, Baldamus CA: Peripheral hemodynamics, blood viscosity and the renin-angiotensin system in hemodialysis patients under therapy with recombinant human erythropoietin. *Contrib Nephrol* 76:292–298, 1989
20. Duke M, Abelman W: The hemodynamic response to chronic anemia. *Circulation* 39:503–515, 1969
21. Heidenreich S, Rahn KH, Zidek W: Direct vasopressor effect of recombinant human erythropoietin on renal resistance vessels. *Kidney Int* 39:259–265, 1991

22. Tepel M, Wischniowski H, Zidek W: Erythropoietin increases cytosolic free calcium concentration and thrombin induced changes in cytosolic free calcium in platelets from spontaneously hypertensive rats. *Biochem Biophys Res Commun* 177:991–997, 1991
23. Miller BA, Bell LL, Lynch CJ, Cheung JY: Erythropoietin modulation of intracellular calcium: A role for tyrosine phosphorylation. *Cell Calcium* 16:481–490, 1994
24. Augustin HG, Kozian DH, Johnson RC: Differentiation of endothelial cells: Analysis of the constitutive and activated endothelial cell phenotypes. *Bioessays* 16:901–906, 1994
25. Dejana E: Endothelial adherens junctions: Implications in the control of vascular permeability and angiogenesis. *J Clin Invest* 98:1949–1953, 1996
26. Furchgott RF: Role of endothelium in responses of vascular smooth muscle. *Circ Res* 53:557–573, 1983
27. Furchgott RF: The role of endothelium in responses of vascular smooth muscle to drugs. *Annu Rev Pharmacol Toxicol* 24:175–197, 1984
28. Palmer RM, Ferrige AG, Moncada S: Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature* 327:524–526, 1987
29. De Mey JG, Vanhoutte PM: Heterogeneous behavior of the canine arterial and venous wall. Importance of the endothelium. *Circ Res* 51:439–447, 1982
30. Harder DR: Pressure-induced myogenic activation of cat cerebral arteries is dependent on intact endothelium. *Circ Res* 60:102–107, 1987
31. Katusic ZS, Shepherd JT, Vanhoutte PM: Endothelium-dependent contraction to stretch in canine basilar arteries. *Am J Physiol* 252:H671–H673, 1987
32. Yanagisawa M, Kurihara H, Kimura S, Tomobe Y, Kobayashi M, Mitsui Y, Yazaki Y, Goto K, Masaki T: A novel potent vasoconstrictor peptide produced by vascular endothelial cells. *Nature* 332:411–415, 1988
33. Clozel M, Fischli W: Human cultured endothelial cells do secrete endothelin-1. *J Cardiovasc Pharmacol* 13(Suppl 5):S229–S231, 1989
34. Marsden PA, Danthuluri NR, Brenner BM, Ballerman BJ, Brock TA: Endothelin action on vascular smooth muscle involves inositol triphosphate and calcium mobilization. *Biochem Biophys Res Commun* 158:86–93, 1989
35. Highsmith RF, Pang DC, Rapoport RM: Endothelial cell derived vasoconstrictors: Mechanism of action in vascular smooth muscle. *J Cardiovasc Pharmacol* 13(Suppl 5):S36–S44, 1989
36. Noguchi CT, Bae KS, Chin K, Wada Y, Schechter AN, Hankins WD: Cloning of the human erythropoietin. *Blood* 78:2548–2556, 1991
37. Bloch KD, Friedrich SP, Lee MN, Eddy RL, Shows TB, Quertermous T: Structural organization and chromosomal assignment of the gene encoding endothelin. *J Biol Chem* 264:10851–10857, 1989
38. Marsden PA, Heng HHQ, Scherer SW, Stewart RJ, Hall AV, Shi XM, Tsui LC, Schappert KT: Structure and chromosomal localization of the human constitutive endothelial nitric oxide synthase gene. *J Biol Chem* 268:17478–17488, 1993
39. Carlini RG, Dusso AS, Obialo CI, Alvarez UM, Rothstein M: Recombinant human erythropoietin (rHuEPO) increases endothelin-1 release by endothelial cells. *Kidney Int* 43:1010–1014, 1993
40. Dusting GJ: Nitric oxide in cardiovascular disorders. *J Vasc Res* 32:143–161, 1995
41. Wennmalm A: Endothelial nitric oxide and cardiovascular disease. *J Intern Med* 235:317–327, 1994
42. Decaterina R, Libby P, Thannickal VJ, Peng HB, Rajavashisth TB, Gimbrone MA Jr, Shin WS, Liao JK: Nitric oxide decreases cytokine-induced endothelial activation: Nitric oxide selectively reduces endothelial expression of adhesion molecules and proinflammatory cytokines. *J Clin Invest* 96:60–68, 1995
43. Broudy VC, Lin N, Egrie J, De Huen C, Weiss T, Papayannopoulou T, Adamson JW: Identification of the receptor for erythropoietin on human and murine erythroleukemia cells and modulation by phorbol ester and dimethyl sulfoxide. *Proc Natl Acad Sci USA* 85:6513–6517, 1988
44. Todokoro K, Kanazawa S, Amanuma H, Ikawa Y: Specific binding of erythropoietin to its receptor on responsive mouse erythroleukemia cells. *Proc Natl Acad Sci USA* 84:4126–4130, 1987
45. Fraser JK, Nicholls J, Coffey C, Lin FK, Berridge MV: Downmodulation of high-affinity receptors for erythropoietin on murine erythroblasts by interleukin 3. *Exp Hematol* 16:769–773, 1988
46. Krantz SB, Sawyer ST, Sawada KI: Purification of erythroid progenitor cells and characterization of erythropoietin receptors. *Br J Cancer* 58(Suppl 9):31–35, 1988
47. Nagao M, Matsumoto S, Masuda S, Sasaki R: Effect of tunicamycin treatment on ligand binding to the erythropoietin receptor: Conversion from two classes of binding sites to a single class. *Blood* 81:2503–2510, 1993
48. Flamme I, Risau W: Introduction of vasculogenesis and hematopoiesis in vitro. *Development* 116:435–439, 1992
49. Asahara T, Murohara T, Sullivan A, Silver M, van der Zee R, Li T, Witzenbichler B, Schatteman G, Isner JM: Isolation of putative progenitor endothelial cells for angiogenesis. *Science* 275:964–967, 1997
50. Youssoufian H, Longmore G, Neumann D, Yoshimura A, Lodish HF: Structure, function and activation of the erythropoietin receptor. *Blood* 81:2223–2236, 1993
51. Neumann D, Wikstrom L, Watowich SC, Lodish HF: Intermediates in degradation of the erythropoietin receptor accumulate and are degraded in lysosomes. *J Biol Chem* 268:13639–13649, 1993
52. Cines DB, Pollak ES, Buck CA, Loscalzo J, Zimmerman GA, McEver RP, Pober JS, Wick TM, Konkle BA, Schwartz BS, Barnathan ES, McCrae KR, Hug BA, Schmidt AM, Stern DM: Endothelial cells in physiology and in the pathophysiology of vascular disorders. *Blood* 91:3527–3561, 1998
53. Levin ER: Endothelins. *N Engl J Med* 333:356–363, 1996
54. Nakamura S, Naruse M, Maruse K, Demura H, Uemura H: Immunochemical localization of endothelin in cultured bovine endothelial cell. *Histochemistry* 94:475–477, 1990
55. Stamler JS, Singel DJ, Loscalzo J: Biochemistry of nitric oxide and its redox-activated forms. *Science* 258:1898–1902, 1992
56. Topper JN, Cai J, Falb D, Gimbrone MA Jr: Identification of vascular endothelial genes differentially responsive to fluid mechanical stimuli: Cyclooxygenase-3, manganese superoxide dismutase, and endothelial cell nitric oxide synthase are selectively upregulated by steady laminar shear stress. *Proc Natl Acad Sci USA* 93:10417–10422, 1996
57. Buemi M, Allegra A, Frisina N: Eritropoietina: Non solo anemia renale. *G Ital Nefrol* 12:9–15, 1995